Generation and functional characterization of a BCL10-inhibitory peptide that represses NF-κB activation

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The molecular complex containing BCL10 and CARMA [CARD (caspase recruitment domain)-containing MAGUK (membrane-associated guanylate kinase)] proteins has recently been identified as a key component in the signal transduction pathways that regulate activation of the transcription factor NF-κB (nuclear factor κB) in lymphoid and non-lymphoid cells. Assembly of complexes containing BCL10 and CARMA proteins relies on homophilic interactions established between the CARDs of these proteins. In order to identify BCL10-inhibitory peptides, we have established a method of assaying peptides derived from the CARD of BCL10 in binding competition assays of CARD–CARD self-association. By this procedure, a short peptide corresponding to amino acid residues 91–98 of BCL10 has been selected as an effective inhibitor of protein self-association. When tested in cell assays for its capacity to block NF-κB activation, this peptide represses activation of NF-κB mediated by BCL10, CARD3 and PMA/ionomycin stimulation. Collectively, these results indicate that residues 91–98 of BCL10 are involved in BCL10 self-association and also participate in the interaction with external partners. We also show that blocking of the CARD of BCL10 may potentially be used for the treatment of pathological conditions associated with inappropriate NF-κB activation.

Key words: BCL10-inhibitory peptide, caspase recruitment domain, caspase recruitment domain-containing membrane-associated guanylate kinase (CARMA), nuclear factor κB (NF-κB).

INTRODUCTION

Over the last few years, a number of adaptor molecules that contain the CARD (caspase recruitment domain) have been identified. The CARD was originally described as a protein-binding motif that interacts with caspase through a CARD–CARD homophilic interaction [1]. However, the CARD has also been found in many adaptor proteins that do not interact with caspases, but mediate the assembly of proteins involved in apoptosis and/or the control of the activation state of the transcription factor NF-κB (nuclear factor κB). Thus it is now well established that CARD-containing proteins play important roles in the assembly and activation of apoptotic and inflammatory complexes, which are mediated by homotypic interactions between CARDs [2].

BCL10 is a CARD-containing protein that was initially identified as a target of the chromosomal translocation t(1;14)(p22;q32), which is found in certain aggressive cases of MALT (mucosa-associated lymphoid tissue translocation) B-cell lymphomas [3,4]. The resulting overexpression of BCL10 leads to constitutive activation of NF-κB, which eventually promotes cell proliferation and tumorigenesis. Several groups independently identified BCL10 as a regulator of apoptosis and as an activator of NF-κB [5,6–10]. Altogether, these studies show that an intact CARD is required for the NF-κB-inducing activity of BCL10, since the point mutations L41Q and G79R introduced in conserved residues of the CARD of BCL10 result in an inactive form of the protein [8,10]. BCL10-knockout mice are immunodeficient because lymphocytes derived from these mice are unable to activate NF-κB, either following antigen receptor stimulation or PMA and ionomycin treatment [11]. In addition, BCL10-deficient mice also exhibit impaired neuronal-tube closure, thus implicating BCL10 not only in lymphocyte activation, but also in neural development [11]. BCL10 interacts with other CARD-containing cytoplasmic molecules, via homophilic interactions mediated by its CARD. These include RIP2 (receptor-interacting protein 2) [12], CARMA [CARD-containing MAGUK (membrane-associated guanylate kinase)] [1] [also known as CARD11 or Bimp (BCL10-interacting MAGUK protein)] [13–15], CARMA2 (also known as CARD14 or Bimp3) [13], CARMA3 (also known as CARD10 or Bimp1) [16,17] and CARD9 [18]. The three CARMA proteins share similar structural motifs, with an N-terminal CARD, followed by a coiled-coil domain, a PDZ domain, an SH3 (Src homology 3) domain and a C-terminal GUK (guanylate kinase)-like domain [13–17]. They display distinct expression profiles, with CARMA1 expressed in haemopoietic cells, CARMA2 in the placenta, and CARMA3 in all non-haemopoietic cells [13–17]. Functionally, CARMA proteins are supposed to function as molecular scaffolds that assist recruitment and assembly of signal transduction molecules. For example, in the TCR (T-cell receptor) signalling pathway, phosphorylated CARMA1 recruits...
BCL10, MALT1 and TRAF6 (tumour-necrosis-factor-receptor-associated factor 6) [18–20]. MALT1 and TRAF6 function as E3 ligases to further induce Lys63-linked polyubiquitination of NEMO (NF-κB essential modulator), leading to activation of the IKK [IκB (inhibitor of NF-κB) kinase] complex [21–24]. Similarly to deficiency of BCL10, genetic disruption of CARMA1 results in severe immunodeficiency, owing to lymphocyte failure to activate NF-κB following antigen receptor-induced activation [14,18–20,24–26]. CARMA proteins interact with BCL10 via their CARD, as a CARD mutant of CARMA1 (L39R) shows no binding to BCL10 [18]. Whereas BCL10 translocation is associated with MALT B-cell lymphomas, missense mutations in the coiled-coil domain of CARMA1 are frequently detected in diffuse large B-cell lymphomas [27].

The complex of proteins that comprises CARMA3, BCL10 and MALT1 appears to play an important role in cells outside the immune system. In fact, previous studies indicate that CARMA3 and BCL10 are implicated in the signal transduction pathways elicited by G-protein-coupled receptors, a large family of cell-surface receptors that regulate cell migration, differentiation, proliferation and survival [28–30]. Specifically, it appears that NF-κB activation induced by G-protein-coupled receptors utilizes a pathway dependent on the molecular complex containing CARMA3 and BCL10.

Altogether, these data indicate that BCL10 represents a valid molecular target for the treatment of pathological conditions associated with inappropriate NF-κB activation, ranging from the MALT B-cell lymphomas with t(1;14)(p22;q32), to the inflammatory disorders involving G-protein-coupled receptors, such as chronic liver injury, atherosclerosis and cardiac hypertrophy [31]. In the present paper, we report the identification of a short BCL10 fragment able to block CARD self-association and prevent activation of the transcription factor NF-κB promoted by BCL10. We also identify residues involved in this activity and thus potentially involved in CARD oligomerization and formation of large protein complexes that mediate activities of BCL10 and of its interacting partners.

MATERIALS AND METHODS

Reagents

N-terminal-protected Fmoc (fluoren-9-ylmethoxycarbonyl)–amino acid derivatives and coupling reagents for peptide synthesis were from Inbio. Peptides were prepared by standard solid-phase synthesis as C-terminally amidated and N-terminally acetylated derivatives following Fmoc chemistry protocols [32]. Reagents for peptide synthesis, solvents and other reagents were all from Sigma–Alrich and LabScan. Anti-BCL10 and anti-CARMA3 antibodies have been generated in our laboratory [2,9,10]. All plasmids and expression vectors used in the present study were generated by standard procedures and confirmed by sequencing [33].

Expression and biotinylation of BCL10-CARD

The doubly mutated C29S/C57S N-terminal region of BCL10, comprising residues 1–107, of BCL10-CARD, was recombinantly expressed in Escherichia coli as a His6-tagged fusion protein. Aliquots of the recombinant BCL10-CARD were biotinylated using the EZ Link NHS-LC-biotin [succinimidyl-6-(biotinamido)hexanoate] reagent (Pierce) according to the manufacturer’s instructions. Biotinylated samples were dialysed against 50 mM sodium acetate buffer (pH 4.0), and protein biotinylation was confirmed by LC (liquid chromatography)–MS analysis ascertaining the increase in molecular mass by 340 Da due to the biotin moiety.

BCL10-CARD self-association and competition ELISA assays

To identify protein regions involved in self-association, the procedure described by Toratore et al. [34] was adopted. To monitor BCL10-CARD self-association, we set up an ELISA-like assay where the protein was adsorbed on the plate surface at different concentrations, whereas the biotinylated variant was utilized to detect the binding. Adsorption was performed in a 96-well plate (Falcon) in 50 mM sodium acetate (pH 4.0) at protein concentrations of 0.12, 0.31 and 0.62 μM overnight at 4°C (100 μl/well). Binding competition assays were carried out by coating the unlabelled BCL10-CARD at a fixed concentration of 0.12 μM and using a fixed concentration of 0.80 μM soluble biotinylated protein (7:1 mol/mol ratio, pre-saturation condition). Peptides contained within the 11 HPLC fractions obtained by trypsin digestion were used at a nominal concentration of 8.0 μM (10:1 ratio with the biotinylated protein).

Cell culture and luciferase assay

HEK (human embryonic kidney)-293 cells and MEFs (mouse embryonic fibroblasts) were grown in complete DMEM (Dulbecco’s modified Eagle’s medium) supplemented with 10% FCS (fetal calf serum) and 100 μg/ml penicillin /streptomycin, and maintained at 37°C with 5% CO2. For plasmid transfection, HEK-293 cells were transfected in 100-mm-diameter Petri dishes by calcium phosphate precipitation. Immunoblot analysis and luciferase tests were performed as described previously [35].

Immunoblot analysis and co-precipitation

Cell lysates were made in lysis buffer (150 mM NaCl, 20 mM Heps, pH 7.4, 1% Triton X-100, 10% glycerol and a mixture of protease inhibitors). Proteins were resolved by SDS/PAGE (10% gel) and were transferred on to a nitrocellulose membrane. Primary antibody incubations were carried out overnight at 4°C in PBS, 0.05% Tween 20 and 2.5% dried non-fat skimmed milk. Secondary antibody incubations were carried out for 1 h at room temperature (25°C) in blocking buffer. Reacting bands were detected using the ECL® (enhanced chemiluminescence) system (GE Healthcare). For co-immunoprecipitation experiments, cells were lysed in lysis buffer and immunocomplexes were bound to Protein A/G, resolved by SDS/PAGE and analysed by immunoblot assay.

RESULTS

Preparation and characterization of BCL10-CARD

BCL10-CARD was expressed as a C29S/C57S isosteric double mutant of the wild-type protein domain (Figure 1) to prevent oligomerization by covalent cysteine cross-linking. The poly-peptide used also contained an N-terminal linker bearing the His6 tag utilized for protein affinity purification. The sequence numbering adopted here is based on that of the native protein domain, ignoring for a matter of clarity the N-terminal fusion tag. The protein was successfully expressed in BL21(DE3) E. coli cells. The protein recovered after the affinity chromatography and gel-filtration purification steps was more than 90% pure and was utilized in all subsequent studies without any further manipulation. The poly-peptide was characterized by LC–MS analysis, confirming purity and the exact molecular mass (results
Identification of BCL10-CARD regions involved in self-association

In order to determine protein regions involved in CARD self-association, peptide fragments generated by trypsin treatment of BCL10-CARD and successively RP (reverse-phase)-HPLC-fractionated were used as inhibitors of protein self-association in a competitive ELISA (see below). For this purpose, a preliminary dose-dependent binding assay was carried out, setting up the optimal conditions for the inhibition test. Figure 2(A) shows the plot of curves relative to the binding assay at different protein concentrations. As shown, at protein coatings of 0.12 and 0.30 μM, the binding is saturated for concentrations of soluble protein higher than approx. 1.5 μM, suggesting that several BCL10-CARD molecules can bind to one coated polypeptide. The Kd value was obtained by averaging the Kd values of the three curves derived by non-linear regression fitting of data (GraphPad Prism, version 4.0) and was estimated to be 1.30 ± 0.16 μM. The binding competition test was performed by coating the protein at a fixed concentration, 0.12 μM, and using the biotinylated variant at 0.80 μM. Competitors were used at a nominal 10-fold excess over the soluble protein. The concentration of peptide fragments was deduced by assuming an initial quantitative protein cleavage by trypsin and a subsequent recovery of 50% by RP-HPLC fractionation.

The LC-MS analysis of fractions showed that protein fragments were distributed as described in Table 1. As can be seen, fractions F1 and F2 did not contain peptides, whereas fractions F3–F8 and fraction F10 contained more than one fragment, while fraction F9 was constituted only by the Leu68–Lys77 fragment. In spite of prolonged reaction times and high temperature (37°C), many fragments contained uncleaved lysine and arginine peptide bonds, suggesting the presence of poorly accessible cleavage sites. Several other fragments, such as the N-terminal Met1–Lys17 bearing the linker tetrapeptide GSEF (Gly-Ser-Glu-Phe) (see Figure 1) and the peptides His98–Lys84 and Thr98–Lys87 were only poorly detected and thus not considered further. For the assay purposes, the freeze-dried fractions were reconstituted in water, properly diluted with buffer and subsequently assayed as competitors in the BCL10-CARD self-association test. Screening results are summarized in Table 1 (rightmost column). As expected, fractions F1 and F2 in which no peptides were detected, did not provide binding reduction. Among the remaining fractions, only fraction F8, containing the peptides BCL10-(91–98) (sequence TQNFLIQK, abundance 63%), and the homologue BCL10-(89–98) (sequence EKTQNFLIQK, abundance 37%), produced a relevant effect of binding reduction (approx. 50%). The sequence EKTQNFLIQK, which, compared with the most abundant residues 91–98 fragment, only contained two additional residues at the N-terminus, was not considered for further investigations, whereas the peptide BCL10-(91–98) (Peptide I, Table 2) was readily re-prepared by chemical synthesis as an amidated C-terminal and acetylated N-terminal variant. To also investigate the influence of the structured flanking region of the selected peptide on CARD self-association, we designed and synthesized two additional BCL10 peptides containing the residues 91–98 stretch at the C-terminus. These peptides, termed Peptide II and Peptide III, contained residues 78–98 and 68–98 respectively (see Tables 1 and 2). A model for BCL10-CARD was retrieved from the ModBase server of the University of California at San Francisco (http://modbase.ccombio.ucsf.edu/) and visualized using WebLab ViewerPro software, version 3.7 (Molecular Simulations). This model was used to localize the putative structure of the selected peptide within the CARD framework. It was obtained by using the X-ray crystallographic structure of the apoptotic protease-activating factor 1 CARD (PDB code: 1CY5 [36]) as a template, and, despite the very low sequence identity (17%), has a very low E-value (2 × 10−8) and a model score of 0.99. On examination of this putative model, it can be seen that residues 91–98 are inside the modelled sequence and virtually correspond to the CARD fifth and the sixth helices, and residues 68–98 correspond to the protein region including helices 5 and 6, the loop between helix 5 and helix 4 and part of helix 4 (see also Figure 1). The
Table 1  Tryptic BCL10-CARD peptide fragments as identified by LC–tandem MS analysis

Relative distributions of fragments (middle column) were calculated by comparing area integration of extracted ion peaks from a given fragment taken from the different fractions. The relative composition within each fraction was derived by comparing area integrations of extracted ion peaks of all the fraction components (see also [34]).

<table>
<thead>
<tr>
<th>Fraction</th>
<th>BCL10-CARD domain fragments</th>
<th>Relative distribution of fragments (%)</th>
<th>Relative composition (%)</th>
<th>Extent of inhibition (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>F1</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
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<tr>
<td>F2</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
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<td>Ile45–Arg49</td>
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<td>20</td>
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<td>Ile32–Arg36</td>
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<td></td>
<td>Glu60–Arg68</td>
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<td>0</td>
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<td>Lys51–Arg53</td>
<td>100</td>
<td>41</td>
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<td></td>
<td>Val35–Lys31</td>
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<td>Glu89–Lys98</td>
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<td></td>
<td>Thr91–Lys98</td>
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<td>F10</td>
<td>Glu89–Lys115</td>
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<td>24</td>
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</tbody>
</table>

Table 2  Peptides utilized in the present study

Single letter codes are reported. pA stands for the non-natural amino acid β-alanine that was used as a spacer between the Tat and the CARD sequences. Peptides VIII and IX described in the experiment of Figure 6 were also fluoresceinated at their N-terminus with fluorescein-β-Ala.

<table>
<thead>
<tr>
<th>Peptide Name</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>BCL10-(91–98)</td>
</tr>
<tr>
<td>II</td>
<td>BCL10-(78–98)</td>
</tr>
<tr>
<td>III</td>
<td>BCL10-(68–98)</td>
</tr>
<tr>
<td>IV</td>
<td>BCL10-(91–98)-T91A/D92A</td>
</tr>
<tr>
<td>V</td>
<td>BCL10-(91–98)-A93A/F94A</td>
</tr>
<tr>
<td>VI</td>
<td>BCL10-(91–98)-L95A/Q96A</td>
</tr>
<tr>
<td>VII</td>
<td>BCL10-(91–98)-Q97A/K98A</td>
</tr>
<tr>
<td>VIII</td>
<td>Tat–BCL10-(91–98)</td>
</tr>
<tr>
<td>IX</td>
<td>Tat–BCL10-(91–98)-T91A/D92A</td>
</tr>
</tbody>
</table>

synthetic Peptides I–III purified to homogeneity by RP-HPLC and characterized by LC–MS, were utilized in a dose–response binding competition assay under the same conditions reported previously for the screening of protein fragments (coating 0.12 μM; biotinylated protein 0.80 μM). The synthetic peptides were utilized at increasing concentrations between 0.03 and 15 μM, and the results are reported in Figure 2B. The results show that the shorter BCL10-(91–98) strongly reduces protein self-association in a dose-dependent fashion, whereas, unexpectedly, the longer Peptides II and III, although containing this active sequence, interfered only slightly with the interaction. In particular, Peptide I inhibited the protein association by 90% at the highest dose of 15 μM and exhibited an IC50 of 70 nM. In contrast, Peptides II and III exhibited an efficacy between 30 and 40% only, with no further effects at higher concentrations (see Figure 2B). This result was suggestive of a tendency of peptides to self-aggregate; thereby, to investigate this issue further, we carried out SEC (size-exclusion chromatography) experiments in order to determine the apparent molecular mass of the synthetic peptides. Repeated analyses of Peptide I and Peptide II on to the SEC column at increasing concentrations (see Table 3) demonstrated that, whereas the BCL10-(91–98) peptide appeared invariably monomeric, the BCL10-(78–98) variant eluted according to a dimer even at the lowest concentration of 5 μM. This property, together with the incapability to efficiently suppress the CARD BCL10 self-association at concentrations higher than ∼1 μM, suggests that the presence of residues 78–90 may favour the formation of peptide oligomers wherein amino acids 91–98, required for binding to the CARD, are not accessible. Next, we explored single residues within the BCL10-(91–98) peptide...
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Figure 3 Dose-dependent competitive ELISA with the synthetic Peptides I, IV, V, VI and VII

Peptides IV–VII are doubly mutated synthetic variants of the most active residues 91–98 fragment. B/B0, relative absorbance.

involved in protein–protein interactions, and, for this purpose, we designed and synthesized four mutated variants of Peptide I (see Table 2) where adjacent residue pairs were replaced by alanine residues. These molecules, named as reported in Table 2 as Peptides IV, V, VI and VII, were again tested in a dose–response assay along with the parent BCL10-(91–98) and results are summarized in Figure 3. As shown, Peptides I, V, VI and VII still reduced the BCL10-CARD self-association, whereas Peptide IV, with Thr91 and Gln92 changed to alanine, was almost ineffective even at the highest dose (15 μM). Notably, Peptide VI, having residues Leu95 and Ile96 changed to alanine residues, exhibited only a reduced efficacy, being able to lessen the interaction to 50% only at the highest concentration. Also Peptide V, with substitutions of alanine residues for Asn93 and Phe94, had an efficacy ten times lower (≈1 μM) than Peptides I and VII. Altogether, these data suggest that residues 91–98 of BCL10 containing Thr91, Gln92, Leu95 and Ile96 may represent key contact points between protein monomers.

In vivo effect of the BCL10 peptide

To confirm further the efficacy of the selected peptide to block self-association between CARDs of BCL10 and possibly the association between BCL10 and other protein partners, we planned cellular experiments using Tat (transactivator of transcription)-conjugated peptide variants to allow cell entry [37]. Peptides were also labelled with fluorescein to confirm and to quantify peptide entry. As a first step, we assessed whether the new fluorescent Tat-conjugated Peptide VIII containing the active sequence TQNFLIQK was still capable of disrupting the BCL10-CARD self-association. We therefore prepared HEK-293 cells co-transfected with plasmids encoding two tagged versions of BCL10-(1–127) bearing the HA (haemagglutinin) and the FLAG epitopes. Then, lysates from transfected cells were immunoprecipitated with anti-FLAG antibodies either in the absence or presence of increasing concentrations of Peptide VIII and the co-precipitating HA-tagged BCL10-(1–127) was detected by immunoblot assay. Peptide VIII repressed self-association of BCL10-(1–127) in a dose-dependent manner and, at 100 μM, the binding was totally abolished (Figure 4A).

Figure 4 Peptide VIII inhibits CARD-mediated interactions

(A) In vitro inhibition of BCL10-CARD (residues 1–127) self-association in lysates of BCL10-overexpressing cells. Two differentially tagged protein constructs were utilized in this assay. Binding inhibition of 50% was roughly achieved by a concentration of Peptide VIII of ~5 μM. (B) Inhibition of BCL10/CARMA3 CARD association. HEK-293 cells were transfected with BCL10-CARD or CARMA3-CARD and 24 h later, cell lysates were pre-treated for 1 h with PBS or with the indicated concentration of Peptide VIII or Peptide IX. Samples were pooled and immunoprecipitated with anti-FLAG beads overnight. Immunocomplexes were then washed, resolved by SDS/PAGE and analysed by Western blotting. (C) Association of BCL10 with MALT1 is unaffected by Peptide VIII. HEK-293 cells were treated with the indicated concentration of Peptide VIII or Peptide IX or left untreated. Cells were left untreated or stimulated with PMA and ionomycin (Iono) for 40 min to induce NF-κB activation and were then lysed. Samples were immunoprecipitated with anti-BCL10 beads overnight, washed, resolved by SDS/PAGE and analysed by Western blotting. IP, immunoprecipitation; WB, Western blotting.
not the control Peptide IX, also repressed association of BCL10 with the CARD of CARMA3 (Figure 4B), although at higher concentrations (Figure 4B). We also assessed the effect of Peptide VIII on the pre-formed complex containing BCL10 and MALT1. This analysis was conducted on endogenous proteins, rather than overexpressed molecules. As shown in Figure 4(C), association of BCL10 with endogenous MALT1 was unaffected by the presence of Peptide VIII.

To assess peptide entry, HEK-293 cells were treated for 5 h at room temperature with different concentrations of FITC- and Tat-conjugated Peptides VIII and Peptide IX (used as a control). Samples were then fixed and analysed using fluorescence microscopy. Under these conditions, both peptides appeared uniformly distributed in HEK-293 cells at 200 μM (Figure 5A). Cytosfluorimetry experiments also confirmed peptide entry in the cells (Figure 5B). In addition, the peptides were not toxic for the cells at the concentration and time periods used for the study (Figure 5C).

To analyse the effect of Peptide VIII in an in vivo assay, we transiently transfected HEK-293 cells with an expression plasmid encoding the full-length BCL10, along with a luciferase reporter plasmid for NF-κB. After 16 h, cells were treated with both Peptide VIII and Peptide IX (used as a control) for 5 h and luciferase activity was determined. The results of these experiments, shown in Figure 6(A), indicated that Peptide VIII repressed, in a dose-dependent manner, the activation of NF-κB promoted by BCL10 overexpression with a maximum efficacy of approx. 50% at a dose of 50 μM. In the same experiment, treatment of cells with the control Peptide IX had no effect on NF-κB activity. In addition, Peptide VIII had no effect on NF-κB activation elicited by expression of p65, indicating that its inhibitory effect is exerted at an upstream level (Figure 6B).
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Figure 6 Selective inhibition of BCL10-induced NF-κB activation by Tat–Peptide VIII

HEK-293 cells were transiently co-transfected with an expression vector encoding (A) BCL10 or (B) p65, together with NF-κB–luciferase and β-galactosidase reporter vectors. At 16 h after transfection, cells were treated for 5 h with Tat–Peptide VIII and Tat–control (Peptide IX), at the indicated concentrations. Results shown represent relative luciferase activity normalized to β-galactosidase activity and are representative of six independent experiments performed in triplicate.

BCL10 and CARMA3 have been implicated in LPA (lyso-phosphatidic acid)-induced NF-κB activation in MEFs [28–30]. Since PKC (protein kinase C) activity is required for LPA-induced NF-κB activation, we tested whether Peptide VIII inhibited activation of NF-κB following PKC activation. For this purpose, an NF-κB–luciferase reporter plasmid was transfected into HEK-293 cells, and cells were then left untreated or stimulated with ionomycin, a calcium ionophore, plus PMA, a surrogate of 1,2-diacylglycerol, the natural activator of conventional and novel PKCs. In agreement with the previous results, treatment of cells with Peptide VIII, but not with the control Peptide IX, repressed the activation of NF-κB following PKC activation in a time- and dose-dependent manner (Figure 7).

DISCUSSION

Originally identified as a protein–protein interaction motif present in signalling molecules involved in the activation of apoptotic and pro-inflammatory caspases, CARD motifs also function in caspase-unrelated signalling pathways, by mediating CARD–CARD association between CARD-containing binding partners

[1,2]. BCL10 was initially identified in a subset of MALT B-cell lymphomas with t(1;14)(p22;q32) [3,4]. This 233-amino-acid protein is ubiquitously expressed and contains an N-terminal CARD. Importantly, BCL10−/− lymphocytes show an absence of NF-κB activation following antigen receptor stimulation or PMA/ionomycin-induced cell activation [9], which results in severe immunodeficiency. A subfamily of MAGUK proteins bind to and co-operate with BCL10 in promoting activation of NF-κB [13–17]. These proteins, which include CARMA-1, -2 and -3, function as molecular scaffolds and contain a PDZ domain, an SH3 domain and a GUK-like domain [13–17]. Association of these proteins with BCL10 is mediated by a N-terminal CARD, which interacts specifically with the CARD of BCL10, and this interaction is necessary for their function. Accordingly, the experimental hypothesis addressed in the present study is that the disruption of the pattern of interactions involving the CARD of BCL10 should down-regulate BCL10-dependent NF-κB activation.

Given these premises, knowledge of regions involved in protein–protein interactions becomes of utmost importance for the design of BCL-10-targeted NF-κB inhibitors. Hence, by means of a procedure described in a previous paper [34], we have fragmented the CARD of BCL10 with trypsin, and the peptides obtained following HPLC fractionation have been used as competitors in a CARD–CARD interaction assay. Using this
method, we have isolated a fragment corresponding to residues 91–98 of the protein (Peptide I) that strongly abolishes protein self-association both in vitro and in a BCL10-overexpressing cell line. Noticeably, Peptide I has an IC₅₀ of ∼ 70 nM, although when it is fused to the basic region of the Tat protein (Peptide VIII) it is much less effective, and has an IC₅₀ of only ∼ 5 μM (see Figure 4). This result can be imputed to both a reduced affinity of the Tat-conjugated peptide for the protein domain and to a higher stability of the complexes formed by BCL10 in the presence of other proteins. This hypothesis is confirmed by the cellular assays, where a concentration of ∼ 50 μM is needed to block NF-κB activation by approx. 70 % (see Figures 6, 7A and 7B). Importantly, the loss of activity of Peptide VIII is not due to peptide degradation within the cell medium, as it appeared to be highly stable when in contact with serum for over 20 h (results not shown). In addition, Peptides II and III, designed to account for adjacent CARD helices and containing the wild-type residues 91–98 at the C-terminus, were poorly effective in reducing the BCL10-CARD self-association in vitro. This behaviour can be explained by the propensity of the longer polypeptides to self-aggregation and by a consequent inaccessibility of contact residues even at low concentrations. This hypothesis is supported by the observation that the residues 78–98 segment is dimeric even at 5 μM and we expect a similar dimeric structure also for the peptide consisting of residues 68–98. Indeed, the oligomeric organization of these peptides reflects the CARD propensity to self-associate and corroborates the view that recognition is mediated by the C-terminal helix of the globular domain [38]. However, the possibility that other residues within the stretch of residues 78–90 mediates the self-recognition or that the longer peptides probably adopt a conformation which is closer to that within the full-length CARD cannot be ruled out. Our biochemical data also provide the evidence that the side chains of Thr⁶⁸, Gln⁹², Leu⁹⁵ and Ile⁹⁶ are implicated in the pattern of interactions involving the CARD self-recognition of BCL10 and the incapability of the T91A/Q92A synthetic mutant to activate NF-κB even after PMA/ionomycin stimulation strengthens this view and suggests a very critical role for these amino acids in mediating CARD–CARD contacts. Notably, residues 91, 92, 95 and 96, given their alternate distribution along the sequence, could be part of an extended surface on a side of the putative helix 6.

It is well known that BCL10 interacts with CARMA1 and MALT1 to form a large protein complex known as CBM (CARMA1–BCL10–MALT1), which is the ultimate effector of BCL10 activity [38]. Several studies have reported direct interactions between the CARDs of BCL10 and CARMA1 and between the Ig-like domain of MALT1 and residues 107–119 of BCL10. Very recently, it has been found that residues from the putative helix 5 of the BCL10 CARD (Asp⁸⁰ and Glu⁸⁴) mediate the binding to the DD (death domain) of MALT1 and that they are essential for the downstream NF-κB activation; furthermore, it has been demonstrated that mutations within the sixth helix (affecting Gln⁹², Leu⁹⁵ and Ile⁹⁶) preclude this binding by seemingly altering the CARD structural stability [39]. These findings essentially agree with our data as they fit with a model of multiple interactions between CARDs and between CARD and the MALT1 DD, whereby helix 5 mediates the recognition with MALT1 and residues from the adjacent helix 6 are involved in CARD self-association. Importantly, data also suggest that residues 91 and 92 of BCL10 might have a role in the recognition of CARMA3 and therefore for the G-protein-coupled-receptor-mediated NF-κB activation, although a higher peptide concentration is required to observe this effect. Compounds targeting this BCL10 region could down-regulate activation of the transcription factor via multiple pathways, thus resulting much more effective in suppressing cell growth. NF-κB is an obvious pharmacological target for treatments aimed at blocking the inflammatory response in instances where this process becomes chronic or dysregulated, and a variety of anti-inflammatory agents inhibit the NF-κB pathway. However, a major difficulty in the design of these drugs is that it may not be feasible to block the NF-κB pathway for prolonged periods, since NF-κB plays an important role in the maintenance of host defence responses. Recently, it has been shown that pathway-specific NF-κB inhibition could be obtained by targeting transcriptional regulators of NF-κB [40]. In this context, the present study offers the proof of concept that blocking the CARD of BCL10, and possibly the association with other interacting partners, is an effective way to selectively decrease NF-κB activation and can be a valuable therapeutic approach for the treatment of specific pathological conditions associated with inappropriate NF-κB activation.

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